Vol. 126, No. 1, 1985 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 16, 1985 Pages 163-169

PHOTOAFFINITY LABELLING OF ANDROGEN RECEPTORS WITH 17β-HYDROXY-17α-[^{3}H]METHYL-4,9,11-ESTRATRIEN-3-ONE

A.O. Brinkmann, G.G.J.M. Kuiper, W. de Boer, E. Mulder
and H.J. van der Molen

Department of Biochemistry II,
Division of Chemical Endocrinology,
Erasmus University Rotterdam, P.O. Box 1738,
3000 DR Rotterdam, The Netherlands

Received November 21, 1984

The synthetic androgen 17β-hydroxy-17α-[^{3}H]methyl-4,9,11-estratrien-3-one (R1881) has been used as photoaffinity label to characterize androgen receptors in calf uterus and rat prostate. Polyacrylamide gel electrophoresis under denaturing conditions showed that the DNA-binding form of the androgen receptor in calf uterus cytosol is a protein with a molecular mass of 98 kD. In rat prostate cytosol an androgen receptor with a molecular mass of 46 kD could be photoaffinity labelled with R1881. The photoaffinity labelling procedure described here provides a method for studying the hormone binding domain of androgen receptors in partial purified preparations.

Affinity labelling of steroid binding proteins (including steroid metabolizing enzymes) is used increasingly during the last few years and can be performed either through electrophilic steroid affinity labels (e.g. halogenated acetoxyderivatives) or through photoactivation of highly conjugated, mostly synthetic steroid ligands [1-5]. Photoaffinity labelling of the progesterone receptor with the synthetic ligand 17,21 dimethyl-19-nor-4,9-pregnadiene-3,20 dione (R5020) has been extensively and successfully studied with receptor preparations from several sources [3,6-10]. In contrast data on affinity labelling of androgen receptors are scarce and have been reported only very recently [2,11,12]. In these studies the labelling of the androgen receptor has been carried out mainly with the low affinity ligand bromoacetoxydihydrotestosterone. The present investigation describes the photoaffinity labelling of androgen receptors with the synthetic ligand 17β-hydroxy-17α-[^{3}H]methyl-4,9,11-estratrien-3-one (R1881) which is a non-metabolizable androgen with a high affinity for the androgen receptor.

0006-291X/85 $1.50

MATERIALS AND METHODS

Materials

17β-hydroxy-17α-[^{3}H]methyl-4,9,11-estratrien-3-one ([^{3}H]-R1881) (87 Ci/mmole), a synthetic ligand with a high affinity for the androgen receptor [13] was obtained from New England Nuclear (F.R.G.). Leupeptin was purchased from Sigma, U.S.A. and phenylmethylsulphonyl fluoride (PMSF) from Merck, F.R.G. Native calf thymus DNA was obtained from Sigma, U.S.A. and was used for the preparation of DNA-cellulose according to the method of Alberts and Herrick [14]. Thiomonoglycerol was from Fluka A.G. 9α-fluoro-11β,21-dihydroxy-16α-,17[methylethylidene bis(oxy)]pregna-1,4-diene-3,20-dione (Triamcinolone acetonide) was obtained from Sigma. Buffer A: 40 mM Tris-HCl, 1 mM EDTA, 10% (w/v) glycerol, and 10 mM thiomonoglycerol; pH 7.4. Buffer B: Buffer A without thiomonoglycerol, but with 10 mM sodiummolybdate and 0.1 mM dipiridyldisulphide; pH 7.4.

Preparation of calf uterine cytosol and labelling with [^{3}H]-R1881

Calf uterine tissue, stored frozen at -80°C, was homogenized after thawing in 4 volumes of buffer B. After an initial centrifugation at 10,000 x g_{av} for 10 minutes to remove the lipid layer and cell debris the 105,000 x g supernatant fraction was prepared and incubated for 2 h at 0°C with 15 nM [^{3}H]-R1881 and 7.5 μM triamcinolone acetonide either in the presence or absence of 3μM dihydrotestosterone.

Cytosol equilibrated with [^{3}H]-R1881 was precipitated with ammoniumsulphate (40% saturation). The precipitate was solubilized in buffer A and subsequently mixed with DNA-cellulose (approx. 500 fmol receptor per ml DNA-cellulose). The mixture was incubated at 4°C for 90 minutes and poured into a small column. After extensive washing of the DNA-cellulose with buffer A, the androgen receptor complex was eluted from the column with buffer A containing 15 mM $MgCl_2$. The pooled DNA-cellulose eluates were used for irradiation.

Irradiation of receptor preparations

Androgen receptor preparations were irradiated with an Osram HBO 100 W/W-2 high pressure mercury lamp. Between the lamp and the receptor preparation a layer of 1 cm saturated $CuSO_4$ solution was positioned. During the irradiation with U.V. light the samples were kept on ice at a distance of approx. 5 cm from the lamp. Irradiation was performed for 10 min. Subsequently irradiated samples were precipitated with 5% trichloroacetic acid overnight at 4°C. The precipitates were washed extensively with ethylacetate until the washes were free of radioactive steroids. The precipitated proteins were solubilized in SDS-sample buffer and used for gel electrophoresis.

Sodium Dodecylsulphate-Polyacrylamide electrophoresis (SDS-PAGE)

SDS-PAGE under denaturating conditions was carried out according to Laemmli [15] using 8% acrylamide gels. After electrophoresis the slabgels were cut in 2 mm slices and counted in an Instagel cocktail (Packard Instruments) after elution of the radioactivity with Soluene 350 (Packard Instruments) for 3 h at 60°C. The following molecular weight markers were used: β-galactosidase (116K) (Sigma) and the BioRad Low Molecular weight protein marker kit. In some experiments a ^{14}C-methylated protein mixture (Amersham, UK) was used as marker set.

Preparation of rat prostate cytosol and labelling with ^{3}H-R1881

Rat (Wistar strain R-Amsterdam) prostate cytosol was prepared in buffer B containing 0.6 mM PMSF and 0.25 mM leupeptin as described previously [16,17]. Irradiation of cytosol was performed as described above prior to fractionation on an anion exchange column. Fractionation was performed with 500 μl samples on a Mono Q anion exchange column (5 x 50 mm) with the Pharmacia FPLC-system. The elution buffer was the same as for the preparation of cytosol. During gradient elution the final buffer contained 0,35 M NaCl. All separations were carried out at 6°C with a flowrate of 1 ml/min at an operating pressure of 2.8 mPA [17]. Peak fractions were collected and precipitated with 5% TCA and processed for SDS-PAGE as described for the calf uterine androgen receptor.

RESULTS AND DISCUSSION

Calf uterine cytosol labelled with [^{3}H]-R1881 was precipitated with ammonium sulphate (40% saturation) and the precipitate was subsequently solubilized and incubated with DNA-cellulose. After elution with 15 mM $MgCl_2$ a 40-fold purified androgen receptor preparation was obtained. This DNA-binding fraction was irradiated with U.V. light and subsequently precipitated with 5% TCA. The profile of radioactivity after SDS-PAGE of the TCA-precipitate is shown in Figure 1. One major peak of radioactivity was detected corresponding to a protein with a molecular mass of approximately 98 kD. The peak of radioactive R1881 could be completely suppressed in a cytosol preparation when an additional 200-fold molar excess of 5α-dihydrotestosterone was present. This finding indicates that the ligand binding sites are saturable and most likely

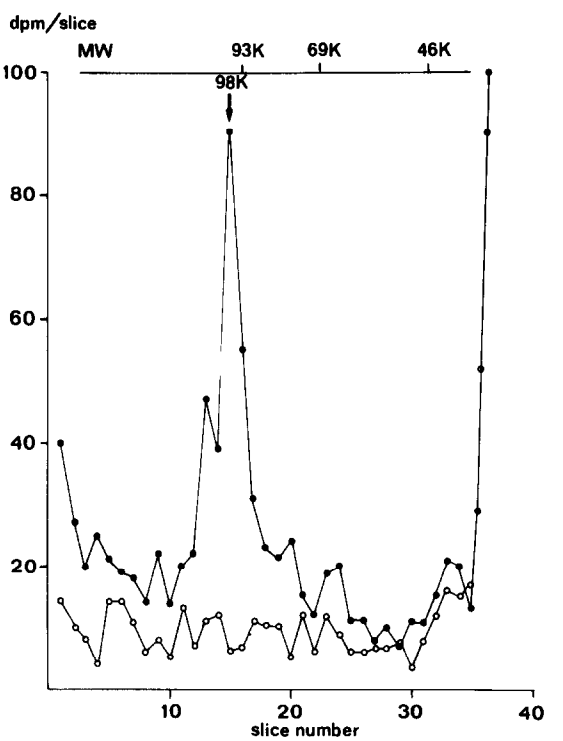

Figure 1
SDS-PAGE profiles of photolysed [^{3}H]-R1881 cytosol preparations from calf uterus in the presence of 7,5 μM triamcinolone acetonide. Irradiation was performed after partial purification with ammonium sulphate precipitation (0-40% saturation) and DNA-cellulose chromatography. • DNA-cellulose bound fraction irradiated in the presence of 15 nM [^{3}H]-R1881; o DNA-cellulose bound fraction irradiated in the presence of 15 nM [^{3}H]-R1881 + 3 μM 5α-dihydrotestosterone.

represent binding to the androgen receptor. The labelling efficiency was approximately 0.2%. At the front of the SDS-PAGE profile photolysed products of R1881 were found.

Since in the calf uterine cytosol progesterone receptor levels are 20-times higher than androgen receptor levels and since the synthetic ligand R1881 has a high affinity for both types of receptor, it was very important to establish that the observed peak of covalently attached R1881 represented the androgen receptor and not the progesterone receptor. The presence of an 98 kD androgen receptor in calf uterine cytosol is supported, however, by the following observations: 1. a 500-fold molar excess of triamcinoloneacetonide was added to the cytosol together with the [^{3}H]-R1881 in order to prevent [^{3}H]R1881 binding to the progesterone receptor [18]; 2. photoaffinity labelling of progesterone receptors either with [^{3}H]-R5020 or with [^{3}H]R1881 resulted in two specifically labelled protein bands appearing at 110 kD and 85 kD respectively 3. a 200-fold molar excess of 5α-dihydrotestosterone completely blocks the covalent attachment of radioactive R1881 to the 98 kD protein, but not to the progesterone receptor. Hence it is concluded therefore that the DNA-binding form of the androgen receptor from calf uterine tissue is a protein with a MW of 98 kD.

Similar studies were performed with rat prostate cytosol. After irradiation of the molybdate stabilized prostate cytosol of castrated rats a fractionation was performed on a Mono Q anion exchange column resulting in a 70-fold purified androgen receptor preparation with a recovery of 75% [17]. The fraction eluted at 0.32 M NaCl was further processed and analysed on SDS-PAGE as described for the calf uterine androgen receptor. In Figure 2 the SDS-PAGE profile is shown. One major peak was detected at 46 kD which could be suppressed by a 200-fold molar excess of 5α-dihydrotestosterone. Molecular mass estimations of the prostate cytosol receptor under non-denaturing conditions with ACA-44 gel chromatography resulted in the same value of 46 kD [17].

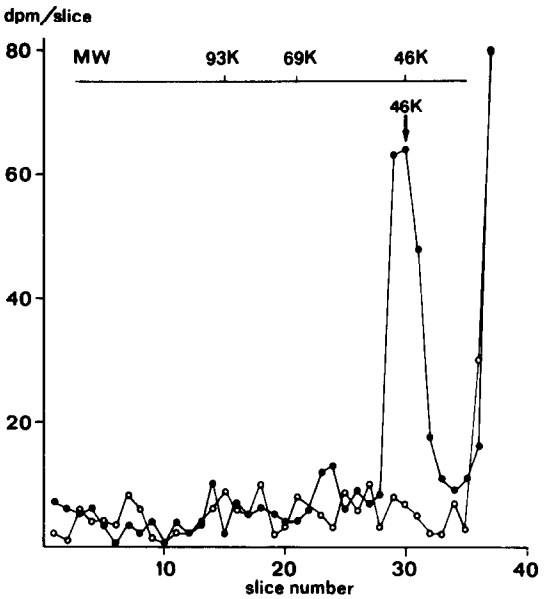

Figure 2
SDS-PAGE profiles of photolysed [^{3}H]-R1881 cytosol preparations from rat prostate after partial purification with FPLC anion exchange chromatography [17]. • cytosol irradiated in the presence of 10 nM [^{3}H]-R1881; o cytosol irradiated in the presence of 10 nM [^{3}H]-R1881 and 2 μM 5α-dihydrotestosterone.

The relationship between the photoaffinity labelled androgen receptor of the prostate and the native form is not certain. During isolation of the receptor the high proteolytic enzyme activities present in prostate cytosol may have affected the molecular size, despite the presence of molybdate (10 mM), PMSF (0.6 mM) and leupeptin (0.25 mM). A larger molecular weight (87 kD) for the prostate androgen receptor from Sprague Dawley rats has been reported recently [2]. In those studies an approx. 500 times purified receptor preparation was used for affinity labelling in combination with the low affinity ligand bromoacetoxydihydrotestosterone.

Several androgen receptor species with smaller molecular weights have been isolated from different androgen target tissues, but their physiological role is not clear. The 46 kD androgen receptor present in the rat prostate for instance showed a high affinity for both RNA and DNA, while a smaller 25 kD receptor form showed affinity only for RNA [20,21]. It is tempting to speculate that in vivo transformation of the androgen receptor to its DNA

binding form might be followed by processing to a form that acquires a high affinity to RNA. Such a process might be useful to remove the receptor from the chromatin binding site through its binding to RNA. More experiments are needed, however, to elucidate the precise steps involved in receptor transformation and processing.

Recently photoaffinity labelling of androgen receptors with R1881 has been questioned by Mainwaring and Randall [19]. In that study, however, the irradiated cytosolic receptor preparations were not further purified, and this might have limited the detection of photolabelled receptors by SDS-PAGE, because of the low efficiency of specific photolabelling and the relatively low concentration of androgen receptors. In the present investigation, a purification of at least 40-fold for both androgen receptor preparations was accomplished and appeared to be essential before analysis on SDS-PAGE.

The present data show that an androgen receptor with a molecular weight of approximately 98 kD can be isolated from a tissue with low endogenous proteolytic enzyme activity (calf uterus). Furthermore this report is the first demonstration of a transformed (DNA-binding) form of the androgen receptor with a molecular mass of approximately 98 kD, which is in the same range as has been reported for transformed receptor forms of the progesterone receptor and the glucocorticoid receptor [8,22]. The photoaffinity labelling technique using [^{3}H]-R1881 as ligand can now be applied as a valuable tool in the analysis of different androgen receptor forms and may facilitate characterization of androgen receptor preparations during purification procedures. Furthermore photoaffinity labelled androgen receptors can be used as probes for the analysis of the steroid binding domain on the receptor.

REFERENCES

1. Warren, J.C. (1973) Receptors for reproductive hormones, 263-288, Plenum Press, New York.
2. Chang, C.H., Lobl, T.J., Rowley, D.R., and Tindall, D.J. (1984) Biochemistry 23, 2527-2533.
3. Dure, L.S. IV, Schrader, W.T., and O'Malley, B.W. (1980) Nature (Lond.) 283, 784-786.
4. Nordeen, S.K., Lan, N.C., Showers, M.O., and Baxter, J.D. (1981) J. Biol. Chem. 256, 10503-10508.
5. Katzenellenbogen, J.A. (1978) Fedn. Proc. 37, 174-178.

6. Smith, R.G., d'Istria, M., and Van, N.T. (1981) Biochemistry 20, 5557-5565.
7. Lamb, D.J., and Bullock, D.W. (1984) Endocrinology 114, 1833-1840.
8. Horwitz, K.B., and Alexander, P.S. (1983) Endocrinology 113, 2195-2201.
9. Gronemeyer, H., Harry, P., and Chambon, P. (1983) Febs Lett. 156, 287-292.
10. Blondeau, J.P., and Baulieu, E.E. (1984) Biochem. J. 219, 785-792.
11. Chang, C.H., Rowley, D.R., Lobl, T.J., and Tindall, D.J. (1982) Biochemistry 21, 4102-4109.
12. Chang, C.H., Rowley, D.R., and Tindall, D.J. (1983) Biochemistry 22, 6170-6175.
13. Bonne, C., and Raynaud, J.P. (1976) Steroids 27, 497-507.
14. Alberts, B., and Herrick, G. (1971) Methods Enzymol. 21, 198-217.
15. Laemmli, U.K. (1970) Nature (Lond.) 227, 680-685.
16. Brinkmann, A.O., Lindh, L.M., Breedveld, D.I., Mulder, E., and Van der Molen, H.J. (1983) Mol. Cell. Endocrinol. 32, 117-129.
17. Brinkmann, A.O., Bolt-de Vries, J., De Boer, W., Lindh, L.M., Mulder, E., and Van der Molen, H.J. (1985) J. Steroid Biochem. in press.
18. Zava, D.T., Landrum, B., Horwitz, K.B., and McGuire, W.L. (1979) Endocrinology 104, 1007-1012.
19. Mainwaring, W.I.P., and Randall, V.A. (1984) J. Steroid Biochem. 21, 209-216.
20. Mulder, E., Vrij, A.A., and Brinkmann, A.O. (1983) Biochem. Biophys. Res. Commun. 114, 1147-1153.
21. Mulder, E., Vrij, A.A., Brinkmann, A.O., Van der Molen, H.J., and Parker, M.G. (1984) Biochim. Biophys. Acta 781, 121-129.
22. Gehring, U. (1983) J. Steroid Biochem. 19, 475-482.